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Activation of AMPK by berberine induces hepatic lipid accumulation by upregulation of fatty acid translocase CD36 in mice

You-Jin Choi [a], Kang-Yo Lee [a], Seung-Hwan Jung [a], Hyung Sik Kim [b], Gayong Shim [a], Mi-Gyeong Kim [a], Yu-Kyoung Oh [a], Seon-Hee Oh [c], Dae Won Jun [d], Byung-Hoon Lee [a,*]

[a] College of Pharmacy, Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Republic of Korea
[b] School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[c] The Division of Natural Medical Sciences, College of Health Science, Chosun University, Gwangju 501-759, Republic of Korea
[d] Internal Medicine, Hanyang University School of Medicine, Seoul 133-791, Republic of Korea

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ABSTRACT

Emerging evidence has shown that berberine has a protective effect against metabolic syndrome such as obesity and type II diabetes mellitus by activating AMP-activated protein kinase (AMPK). AMPK induces CD36 trafficking to the sarcolemma for fatty acid uptake and oxidation in contracting muscle. However, little is known about the effects of AMPK on CD36 regulation in the liver. We investigated whether AMPK activation by berberine affects CD36 expression and fatty acid uptake in hepatocytes and whether it is linked to hepatic lipid accumulation. Activation of AMPK by berberine or transduction with adenoviral vectors encoding constitutively active AMPK in HepG2 and mouse primary hepatocytes increased the expression and membrane translocation of CD36, resulting in enhanced fatty acid uptake and lipid accumulation as determined by BODIPY-C16 and Nile red fluorescence, respectively. Activation of AMPK by berberine induced the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and subsequently induced CCAAT/enhancer-binding protein β (C/EBPβ) binding to the C/EBP-response element in the CD36 promoter in hepatocytes. In addition, hepatic CD36 expression and triglyceride levels were increased in normal diet-fed mice treated with berberine, but completely prevented when hepatic CD36 was silenced with adenovirus containing CD36-specific shRNA. Taken together, prolonged activation of AMPK by berberine increased CD36 expression in hepatocytes, resulting in fatty acid uptake via processes linked to hepatocellular lipid accumulation and fatty liver.

1. Introduction

Berberine is an isoquinoline alkaloid found in many plants, including *Coptis chinensis*, and it is a well-known to have multiple pharmacological activities including anti-microbial and anti-diarrhea activity (Amin et al., 1969; Taylor and Greenough, 1989). Many studies have focused on its effects on the metabolic syndrome, such as obesity and type II diabetes mellitus (Leng et al., 2004; Lee et al., 2006). Berberine activates AMPK indirectly by inhibiting mitochondrial respiratory complex I (Turner et al., 2008). In the liver of ob/ob and db/db mice, AMPK activation by berberine resulted in decreased de novo lipogenesis and increased fatty acid oxidation (Kim et al., 2009). Berberine also can inhibit the proteolytic cleavage and nuclear translocation of SREBP in hepatocyte indicating that berberine have triglyceride and cholesterol lowering effect by AMPK activation (Li et al., 2010; Xia et al., 2011). Clinical trials in patients with type 2 diabetes revealed that berberine reduced plasma glucose and hemoglobin A1c and improved the lipid profile with effects comparable to those receiving metformin (Yin et al., 2008; Zhang et al., 2008).

AMP-activated protein kinase (AMPK) was originally identified as a serine/threonine kinase that phosphorylates and inactivates key mammalian enzymes responsible for fatty acid and cholesterol synthesis, namely acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), respectively (Hardie et al., 1989). The role of AMPK in the regulation of physiological energy metabolism has been studied extensively since then and it is now recognized as the master regulator of energy metabolism in the cell. Upon activation, AMPK causes a cellular metabolic switch from lipogenesis to lipolysis and from hepatic glucose synthesis to muscle glucose utilization. Consequently, AMPK improves insulin sensitivity and glucose homeostasis, making it an attractive therapeutic target for metabolic syndromes, including type 2 diabetes (Hardie, 2013).

The primary role of AMPK in cardiac and skeletal muscle contraction is the production of ATP by the uptake of glucose and fatty acids from circulation, which is achieved by enhanced sarcolemmal translocation of GLUT4 and CD36 from the intracellular vesicles, respectively

* Corresponding author at: College of Pharmacy, Seoul National University, Gwanak-ro 599, Gwanak-gu, Seoul 151-742, Republic of Korea.
E-mail address: lee@snu.ac.kr (B.-H. Lee).

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(Luiken et al., 2003; Russell et al., 1999). Although the exact mechanisms are unclear, a recent study showed that AMPK phosphorylates and inactivates TBC1D class protein, which is known to block AMPK-induced CD36 translocation (Fentz et al., 2015). However, the effects of this mechanism on CD36 status, fatty acid transport and lipid accumulation in the liver require further investigation. The liver is the primary organ for the storage and distribution of surplus nutrients and thus plays an important role in regulating whole body energy homeostasis. Therefore, the major role of AMPK in the liver is to maintain lipid and glucose homeostasis. Basal low levels of CD36 in normal hepatocytes are increased dramatically in animals fed a high-fat diet and in patients with non-alcoholic fatty liver disease (NAFLD) (Buqué et al., 2012; Memon et al., 1999; Zhou et al., 2008). Moreover, hepatic CD36 upregulation is significantly associated with insulin resistance, hyperinsulinemia and increased steatosis in patients with non-alcoholic steatohepatitis and hepatitis C virus infection (Miquilena-Colina et al., 2011). Importantly, overexpression of CD36 in the liver of normal fed, non-metabolically challenged mice increased hepatic fatty acid transport and induced fatty liver (Koonen et al., 2007).

In the present study, we investigated the effects of AMPK activation by berberine in normal mouse hepatocytes and livers in terms of CD36 expression, fatty acid uptake and lipid accumulation. We found that activation of AMPK by berberine or genetic manipulation in hepatocytes increased the expression and translocation of CD36, which resulted in increased fatty acid uptake and lipid accumulation. In addition, hepatic CD36 expression and triglyceride (TG) levels were increased in normal diet-fed mice administered berberine, which was completely prevented upon CD36 silencing with small-hairpin RNA (shRNA).

2. Materials and methods

2.1. Cell culture and chemicals

Human hepatoma cell line, HepG2 (ATCC, Manassas, VA, USA) were maintained in DMEM (GIBCO BRL, Grand Island, NY) containing 10% heat-inactivated FBS (GIBCO BRL) and 1% antibiotic-antimycotic (GIBCO BRL). Mouse hepatocytes were isolated from specific pathogen-free male C57BL/6 mice (20–25 g) by two-step perfusion with calcium and magnesium-free Hanks' salt solution followed by a medium containing collagenase with modifications (LeCluyse et al., 1996). Cells were incubated at 37 °C with the air containing 5% CO_2. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Stock solution of berberine chloride (0.1 M) and AICAR (5-aminoimidazole-4-carboxamide ribonucleotide; 0.1 M) was prepared in DMSO, which was further diluted to the desired concentrations using the cell culture medium.

2.2. Nile red assay and fatty acid uptake assay

Intracellular lipid accumulation was quantified using Nile red, a fluorescent dye that binds to neutral lipids (McMillian et al., 2001). HepG2 cells and primary mouse hepatocytes were seeded in 96 well plates. The cells were stabilized for 24 h and treated with berberine or AICAR for 24 h. On the following day, cells were fixed with 4% paraformaldehyde and stained with 1 μg/ml Nile red solution. The fluorescence was measured with a microplate fluorescence reader (Molecular Devices, Sunny-vale, CA; excitation 488 nm and emission 580 nm) or was observed with an epifluorescence.

Fatty acid uptake was measured using a fluorescent palmitate analog, BODIPY-C16. Stock solutions of BODIPY-C16 (Invitrogen Life Sciences, Carlsbad, CA) were prepared in DMSO. After berberine or AICAR treatment, cells were incubated for 3 min in PBS supplemented with BODIPY-C16 to a final concentration of 100 nM, rinsed with ice-cold PBS, and fixed in ice-cold 4% paraformaldehyde for 10 min. The fluorescence was measured with a microplate fluorescence reader (excitation 500 nm and emission 515 nm) or was observed with an epifluorescence.

2.3. Adenoviral infection and siRNA transfection

A constitutively active mutant of AMPK (Ad-CA-AMPK) and dominant-negative AMPK (DN-AMPK; T172A) plasmid were provided by Dr. Joohun Ha from Kyung Hee University. Ad-CA-AMPK were prepared and purified as described previously (Lee et al., 2003). siRNA for CD36 and C/EBPβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were infected with adenovirus for 6 h and incubated for 48 h after medium change. Transient transfection with DN-AMPK plasmid, si-C/EBPβ and si-CD36 were performed by using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer's protocol.

2.4. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as described with some modifications (Nelson et al., 2006). Briefly, cell lysate was fixed with 1% formaldehyde in PBS and the cross-linking was stopped with 125 mM glycine at room temperature. Chromatin was sheared by sonication and immunoprecipitated with anti-C/EBPβ antibody or non-immune IgG antibody at 4 °C. Immune complexes were collected with protein A-Sepharose beads (Amersham Biosciences). Total DNA was purified by Chelex 100-based method.

The binding of C/EBPβ to the CD36 promoter was detected by PCR. The sequences for primers over −273 to +19 nt of the mouse FAT/CD36 promoter were: forward, 5′ CTGGCCT CTGACTTACTTGGATGGGA 3′, reverse, 5′ GTCCTACACTGCAGTCCTCACTACATA 3′.

2.5. Subcellular fractionation

Cells were washed with cold PBS and collected in hypotonic buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 0.2 mM sodium orthovanadate, 1 mM PMSF) containing 1× protease inhibitor cocktail (Roche, Basel, Switzerland). The cell suspension was incubated on ice for 1 h and homogenized by 50 strokes through a Dounce homogenizer followed by incubation on ice for 30 min. Lysates were centrifuged at 1000*g* at 4 °C for 10 min. The pellet, containing the nuclei and cell debris, was discarded and the supernatant (cytosol fraction) was centrifuged at 1,000,000*g* for 40 min to obtain pellets containing membrane fraction. Pellets were resuspended in lysis buffer containing 1% Triton X-100 and were incubated for 15 min. The suspension was sonicated on ice with 3 pulses (9 s) with 5 s intervals followed by 15 min incubation. The sonicated suspension was centrifuged at 1,000,000*g* for 40 min. The supernatant was considered the membrane fraction.

2.6. Immunofluorescence microscopy

For CD36 immunofluorescence microscopy, cells were washed with PBS and fixed with 3.7% paraformaldehyde at 4 °C for 10 min, and washed again with PBS. The cells were then blocked with 1% BSA at room temperature and incubated with anti-CD36 antibody diluted 1:100 in PBS containing 0.5% BSA at 4 °C for 12 h, followed by sequential incubation with Alexa Fluor® 488 dye-labeled secondary antibody. The specimens were mounted using ProLong® Gold Antifade Reagent (GIBCO BRL) after washing and examined with an epifluorescence microscope.

2.7. Animal treatments

Specific-pathogen-free male C57BL/6 mice (6 weeks old) was purchased from Central Lab. Animal Inc. and allowed ad libitum access to standard chow and tap water. They were kept in temperature-controlled, filter-sterilized animal quarters under a 12 h light:12 h dark

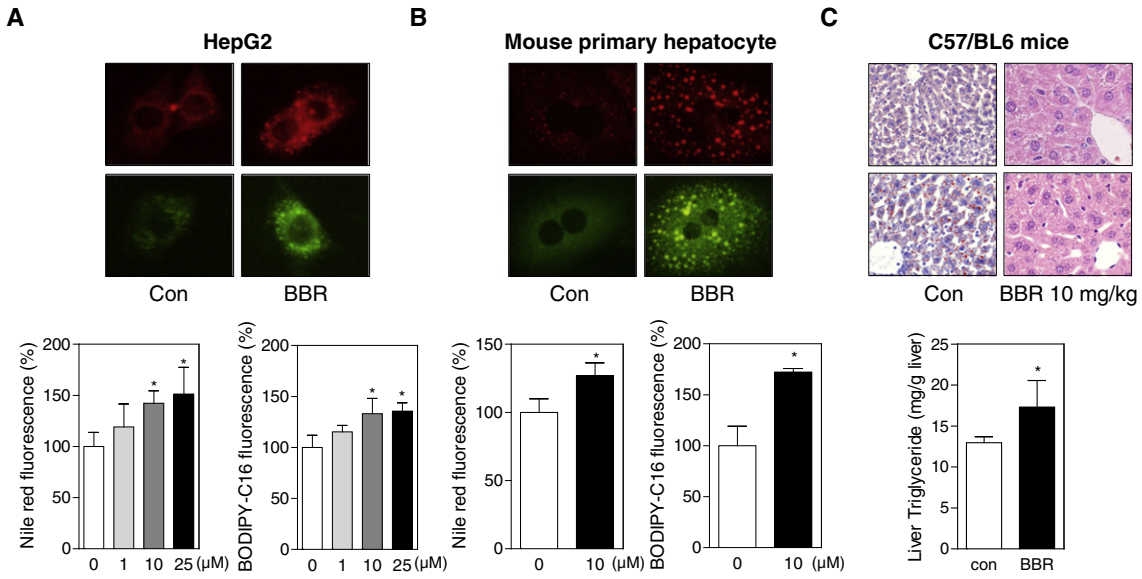

Fig. 1. Berberine increases hepatocellular fatty acid uptake and lipid accumulation. (A–B) HepG2 cells and mouse hepatocytes were incubated with berberine for 24 h. Fatty acid uptake and lipid accumulation were determined by BODIPY-C16 and Nile red fluorescence, respectively. Each bar represents three independent experiments. (C) C57BL/6 male mice were administered berberine (10 mg·kg^{-1}·day^{-1}) intraperitoneally for 7 days. Histological images are shown at original magnification (400×, left panel: Oil Red O; right panel: H&E). Liver triglycerides were measured as described in Section 2 (Materials and methods). Each bar represents three independent experiments or the mean ± SD of four mice. Statistical analysis was performed using Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test where appropriate. * Indicates significance ($p < 0.05$) relative to the control.

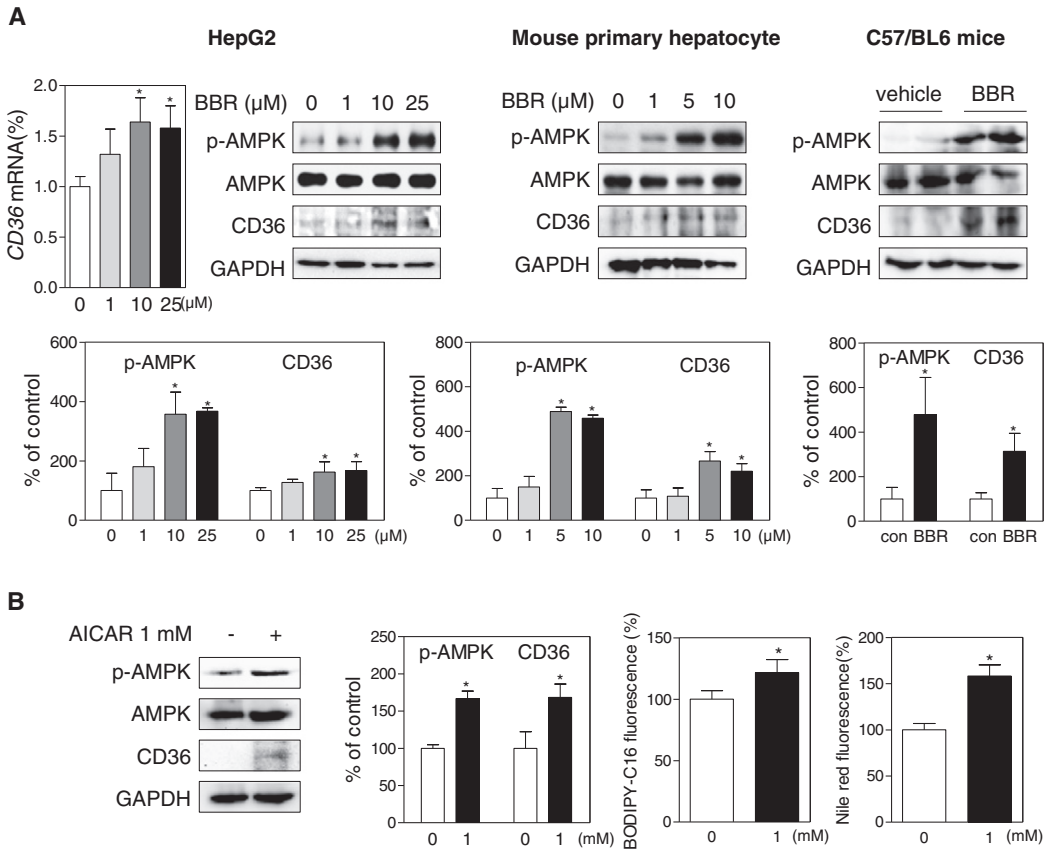

Fig. 2. AMPK activation by berberine increases fatty acid uptake mediated by CD36. (A) HepG2 cells and mouse hepatocytes were incubated with berberine for 24 h. C57BL/6 male mice were administered berberine (10 mg·kg^{-1}·day^{-1}) intraperitoneally for 7 days. (B) HepG2 cells were treated with AICAR (1 mM) for 24 h. mRNA levels of CD36 were measured by quantitative PCR. Fatty acid uptake and lipid accumulation were determined by BODIPY-C16 and Nile red fluorescence, respectively. Total cell lysates and mouse liver homogenates were isolated and Western blots were performed. Band densities were determined using an image analysis system and expressed as percentages of the control. Each bar represents three independent experiments or the mean ± SD of four mice. Statistical analysis was performed using Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test where appropriate. * Indicates significance ($p < 0.05$) relative to the control.

cycle. The use of animals was in compliance with the guidelines established by the animal care committee of our institute. Mice were randomly assigned to four groups. Treatment groups were administered with berberine (10 mg/kg/day; i.p.) for seven days with 1×10^9 plaque-forming units (pfu) of adenovirus containing control (Ad-shmock) or CD36-specific shRNA (Ad-shCD36) twice with 3 days interval according to our preliminary experiments. Control groups were received the same volume of physiologic saline by intraperitoneal injection.

2.8. Histological analysis

A cross section of the left lateral lobe of the liver was fixed in 10% neutral buffered formalin for 24 h. The liver tissues were dehydrated, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E). Immunostaining was performed on formalin-fixed paraffin embedded liver sections from all mice of each group. Preparations were incubated overnight with the primary rabbit antibody sc-9154 (Santa Cruz Biotechnology) diluted to 1:200 at room temperature in humid chambers and were counterstained in hematoxylin. Lipid deposits in liver were visualized by staining with Oil red O. For Oil Red O staining liver tissues were embedded in Tissue-tek OCT compound (Sakura Finetek, Torrance, CA) and frozen cryosections of the tissue (7 μm) were stained with Oil Red O.

2.9. Measurements of serum biochemistry

Serum were separated from blood by low-speed centrifugation (1500*g*, 15 min, 4 °C), transferred into aliquots, and stored at −70 °C until analysis. The serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and cholesterol were monitored by standard clinical chemistry assays on an Automated Chemistry Analyzer (Prestige 24I; Tokyo Boeki Medical System, Tokyo, Japan).

2.10. Determination of serum and liver TG

Total liver lipids were extracted from homogenate prepared from 100 mg of mouse liver using chloroform/methanol mix (2:1, v/v). Serum and liver TG were determined enzymatically using EnzyChrom Triglyceride Assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer's protocol.

2.11. Western blot analysis

Cell pellets and liver homogenates were lysed on ice for 60 min in lysis buffer (120 mM NaCl, 40 mM Tris, pH 8, 0.1% NP-40) and centrifuged at 12,000 rpm for 10 min. Supernatants were collected, electrophoresed on 7–10% SDS-PAGE and transferred onto PVDF membrane (Millipore, Bedford, MA). Western blots were probed with the following antibodies: p-AMPK (Thr172, #2531), AMPK (#2532), p-ERK (#4370), ERK (#4695), and GAPDH (#2118) from Cell Signaling Technology (Beverly, MA), and CD36/FAT (sc-9154), p-C/EBPβ (sc-16993-R) and C/EBPβ (sc-150) from Santa Cruz. Detection was performed by enhanced chemiluminescence western blotting detection reagents (Amersham, Piscataway, NJ).

2.12. Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed using Student's *t*-test or one-way ANOVA where appropriate. Differences between groups were considered to be statistically significant at $p < 0.05$. Multiple comparisons were evaluated by one-way ANOVA analysis of variance followed by Tukey's multiple comparison tests.

3. Results

3.1. Berberine increases hepatocellular fatty acid uptake and lipid accumulation

Berberine is an AMPK activating agent that demonstrates antidiabetic and antisteatotic effects in disease models and patients (Kim et al., 2009; Lee et al., 2006; Yin et al., 2008). Unexpectedly, however, treatment of HepG2 cells and mouse primary hepatocytes with berberine for 24 h increased intracellular lipid accumulation and fatty acid uptake as determined by Nile red and BODIPY-C16 fluorescence, respectively (Fig. 1A and B). Given that the concentration of free fatty acid in culture medium is equivalent to approximately 50 μM based on the calculation (Villard et al., 2011), the facilitated fatty acid uptake from culture medium could induce lipid accumulation in cells. Male C57BL/6 mice were administered berberine (10 mg/kg body weight) intraperitoneally for 7 days. Consistent with the in vitro data, the hepatic TG level was increased significantly in the berberine-treated group as confirmed by oil red O staining (Fig. 1C).

3.2. Berberine increases fatty acid uptake mediated by CD36

Fatty acid transport proteins (FATP2 and 5) and liver-type fatty acid binding proteins (L-FABP) are abundantly expressed in the liver (Musso et al., 2009). Although the basal level of hepatic CD36 is quite low,

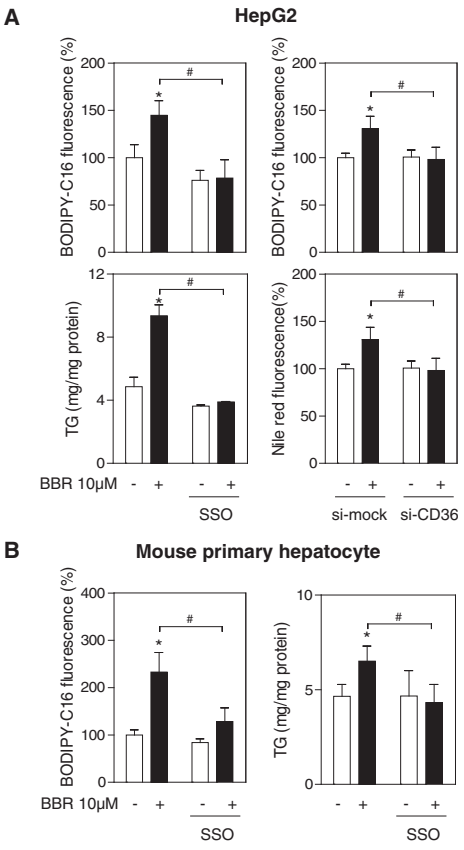

Fig. 3. Pretreatment of sulfo-*N*-succinimidyl oleate (SSO) or transfection with CD36-specific siRNA prevents the effects of berberine on fatty acid uptake and lipid accumulation. (A) HepG2 cells were incubated with SSO for 1 h or transfected with si-CD36 for 48 h. Subsequently, cells were treated with berberine for 24 h. Fatty acid uptake and lipid accumulation were determined by BODIPY-C16 and Nile red fluorescence, respectively. (B) Isolated mouse hepatocytes were treated with SSO and berberine as described above. Each bar represents three independent experiments. * Indicates significance ($p < 0.05$) compared with the control; # indicates significance ($p < 0.05$) compared with berberine treatment using one-way ANOVA followed by Tukey's multiple comparison test.

expression levels are increased substantially in animals fed a high-fat diet and in patients with NAFLD (Koonen et al., 2007; Miquilena-Colina et al., 2011). We investigated whether berberine increases the expression of hepatic fatty acid transporters. Whereas the expression of the major hepatic fatty acid transporters, L-FABP, FATP2 and FATP5, were unchanged (Fig. S1), berberine increased the mRNA and/or protein levels of CD36 in vitro and in vivo (Fig. 2A). To determine whether berberine-induced upregulation of CD36 is mediated by the activation of AMPK, HepG2 cells were treated with AICAR, an AMPK activator, or transduced with Ad-CA-AMPK. Activation of AMPK by AICAR significantly increased the expression of CD36, BODIPY-C16 uptake and Nile red fluorescence (Fig. 2B). Ad-CA-AMPK transduction showed similar results, except for the Nile red fluorescence (Fig. S2). Next, we investigated whether CD36 is responsible for the observed effects. Pretreatment of HepG2 cells with sulfo-*N*-succinimidyl oleate (SSO), a specific inhibitor of CD36, or transfection with CD36-specific siRNA completely eliminated the effects of berberine on fatty acid uptake and lipid accumulation (Figs. 3A and S3). Similar results were obtained in mouse primary hepatocytes (Fig. 3B). These data indicated that berberine induces hepatic lipid accumulation, which is mediated through the upregulation of CD36 and enhanced fatty acid uptake.

3.3. AMPK activation by berberine induces transcriptional activation of CD36 via the ERK-C/EBPβ pathway

AMPK-dependent ERK1/2 activation is responsible for activating the phosphorylation of C/EBPβ (Noh et al., 2011). Thus, we investigated whether prolonged AMPK activation by berberine increased CD36 transcription mediated by the ERK-C/EBPβ pathway. Activation of AMPK by berberine significantly increased the phosphorylation of ERK and C/EBPβ (Fig. 4A). Transfection with DN-AMPK plasmid or pretreatment with an ERK-specific inhibitor, PD98059, blunted the berberine-induced upregulation of CD36 (Fig. 4B and C). AICAR or Ad-CA-AMPK also induced the phosphorylation of ERK and C/EBPβ leading to the CD36 expression (Fig. S4). In addition, we investigated whether AMPK activation enhanced binding of C/EBPβ to the CD36 promoter. Binding of C/EBPβ to the CD36 promoter was significantly increased by berberine as determined by ChIP followed by quantitative PCR (Fig. 5A).

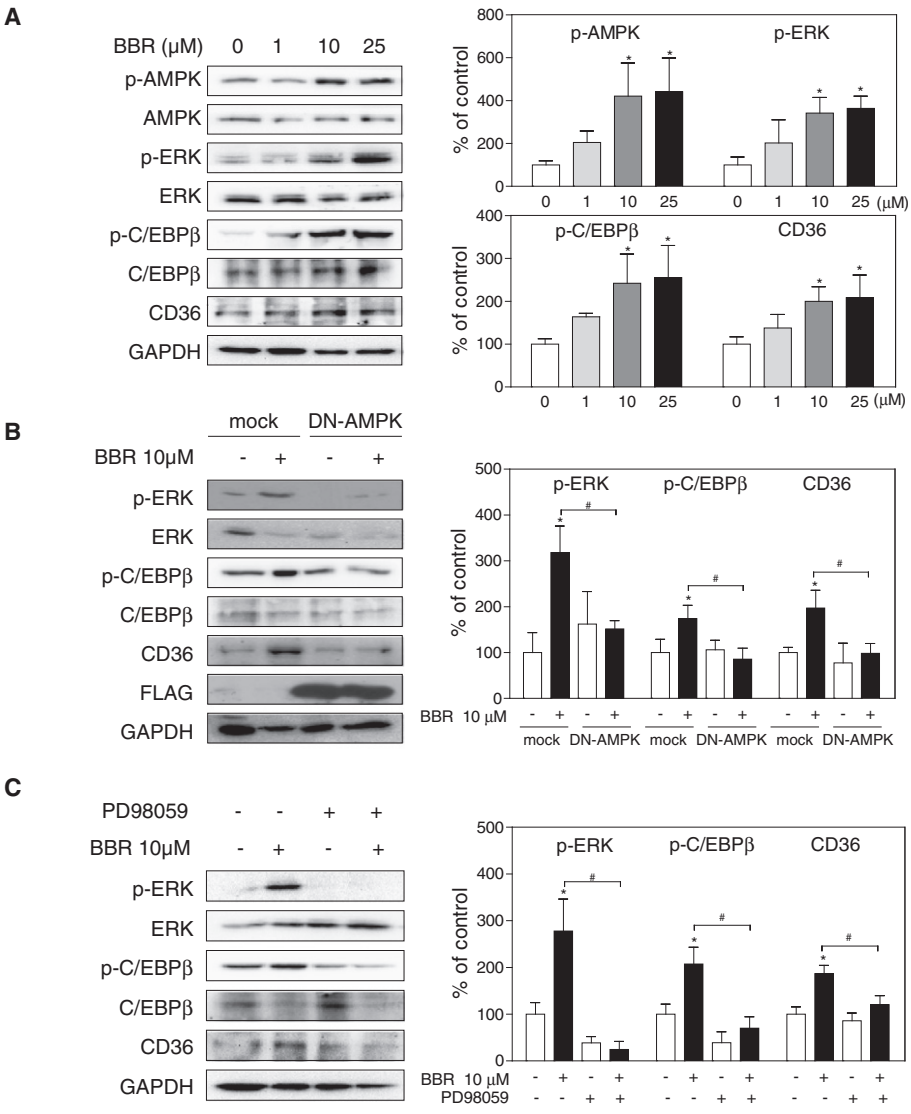

Fig. 4. AMPK activation by berberine increases CD36 transcription mediated by the ERK-C/EBPβ pathway. (A) HepG2 cells were incubated with berberine for 24 h. (B) HepG2 cells were transfected with DN-AMPK plasmid for 48 h. Then, cells were incubated with berberine for 24 h. (C) Cells were incubated with PD98059, a specific inhibitor of ERK, for 1 h. Subsequently, cells were treated with berberine for 24 h. Total cell lysates were isolated, and Western blots were performed. Band densities were determined using an image analysis system and expressed as percentages of the control. Each bar represents three independent experiments. * Indicates significance ($p < 0.05$) compared with the control; # Indicates significance ($p < 0.05$) compared with berberine treatment using one-way ANOVA followed by Tukey's multiple comparison test.

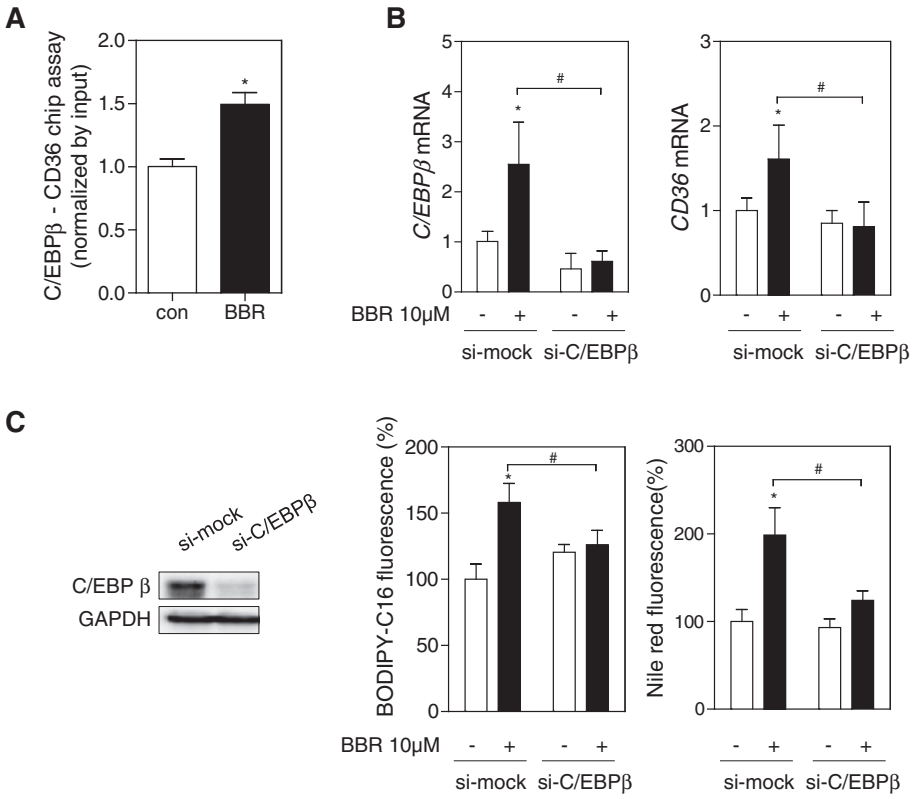

Fig. 5. AMPK activation by berberine increases C/EBPβ binding to the CD36 promoter. (A) ChIP assays were performed using cell lysates treated with berberine. Following immunoprecipitation, DNA was extracted and analyzed for the presence of the C/EBPβ binding region of the CD36/FAT gene by quantitative PCR. Each bar represents three independent experiments. * Indicates significance ($p < 0.05$) relative to the control using student's *t*-test. (B) HepG2 cells were transfected with si-mock or si-C/EBPβ for 48 h. Then, cells were incubated with berberine for 24 h. mRNA levels were measured by quantitative PCR. (C) Fatty acid uptake and lipid accumulation were determined by BODIPY-C16 and Nile red fluorescence, respectively. Each bar represents three independent experiments. * Indicates significance ($p < 0.05$) compared with the control; # Indicates significance ($p < 0.05$) compared with berberine treatment using one-way ANOVA followed by Tukey's multiple comparison test.

Conversely, C/EBPβ RNA interference attenuated berberine-induced CD36 expression, fatty acid uptake and lipid accumulation (Fig. 5B and C).

Contracting muscle increases fatty acid uptake by rapid trafficking of CD36 to the sarcolemma to meet energy needs within several minutes. Therefore, we investigated whether berberine can affect the translocation of CD36 into the hepatic membrane. Berberine treatment for 24 h induced the expression and membrane translocation of CD36, which was confirmed by membrane fraction and fluorescent microscopy (Fig. 6). We also showed that AMPK activation by AICAR induced plasma membrane translocation of CD36 (Fig. S5). These data demonstrated that activation of AMPK by berberine induces transcription of CD36 via the ERK-C/EBPβ pathway.

3.4. Hepatic CD36 expression and TG levels are increased in berberine-treated normal diet-fed mice, but prevented upon CD36 silencing with shRNA

To assess whether increased CD36 expression by berberine is sufficient to develop fatty liver, normal C57BL/6 mice were administered berberine intraperitoneally for 7 days with or without tail vein injection with Ad-shCD36. Fig. 7A shows that Ad-shCD36 injection significantly silenced the hepatic mRNA and protein levels of CD36. Body weight ($p < 0.05$) was slightly decreased by berberine resulting in a higher liver to body weight ratio ($p < 0.05$) both in the sh-mock and shCD36 group (Table 1). AST ($p < 0.05$) was changed significantly by berberine treatment, but remained within the normal range (Table 1). Because AST is not specific to the liver, elevation of AST with normal ALT levels might indicate the extrahepatic effects of berberine. In the sh-mock/berberine group, the total cholesterol level was increased by 28% ($p < 0.05$), which is surprising considering the 'cholesterol-lowering property' of berberine (Kong et al., 2004). The reason for the discrepancy is currently unknown, but it may be attributable to species differences in lipoprotein

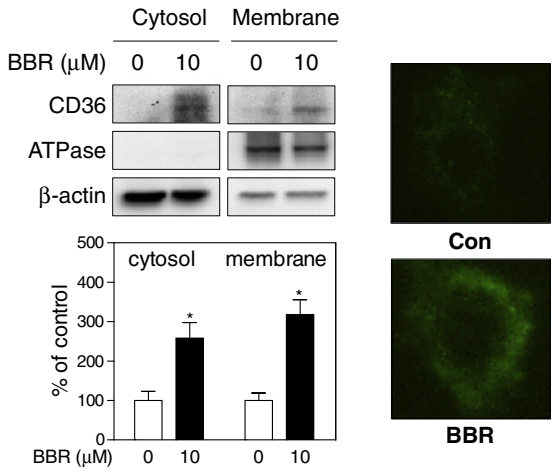

Fig. 6. Berberine induces plasma membrane translocation of CD36. HepG2 cells were incubated with berberine for 24 h. After treatment, total cell lysates were collected and subjected to subcellular fractionation. For immunofluorescence, cells were fixed with 3.7% paraformaldehyde and incubated with anti-CD36 antibody, followed by sequential incubation with Alexa Fluor® 488 dye-labeled secondary antibody. The specimens were observed using an epifluorescence microscope. Band densities were determined using an image analysis system and expressed as percentages of the control. Each bar represents three independent experiments. * Indicates significance ($p < 0.05$) compared with the control using Student's *t*-test.

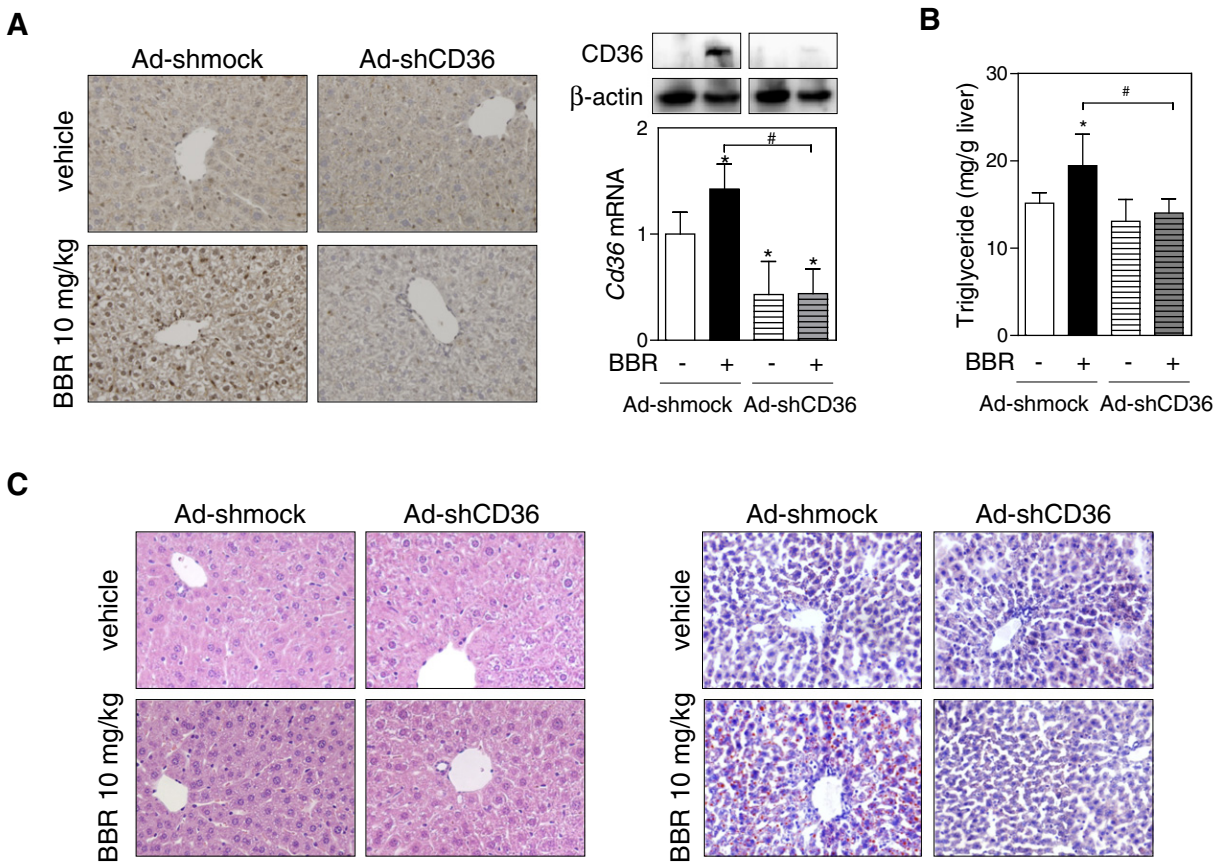

Fig. 7. Hepatic CD36 expression and triglyceride levels are increased in berberine-treated mice on a normal diet, but not after CD36 silencing with shRNA. Male C57BL/6 mice were injected with adenovirus containing control or CD36-specific shRNA via tail vein and followed by intraperitoneal administration of berberine for 7 days. (A) Western blot analysis with a specific antibody was used to examine the protein levels in mouse liver homogenates. CD36 mRNA levels were measured by quantitative PCR. The relative gene levels are represented as fold change compared to the control. (B) Liver triglycerides were measured as described in Section 2 (Materials and methods). (C) Histological images are shown at original magnification (400×, left panel: H&E; right panel: Oil Red O). Each bar represents the mean ± SD of 7 mice. * Indicates significance ($p < 0.05$) compared with the control group; # indicates significance ($p < 0.05$) compared with berberine treatment using one-way ANOVA followed by Tukey's multiple comparison test.

constituent. The major lipoprotein in mice is HDL (Ellis et al., 2013). High dose of berberine administration improved serum lipid profiles in mice by decreasing LDL levels, but total cholesterol levels were increased with the elevation of HDL levels (Chueh and Lin, 2011).

Liver TG was increased by 30% in the sh-mock/berberine group, but was decreased to the control level in sh-CD36/berberine group (Fig. 7B). Hepatic TG was decreased slightly in sh-CD36/vehicle group but was not statistically significant. Oil Red O staining of liver sections confirmed these results (Fig. 7C). Western blot analysis of the liver tissue indicated that the phosphorylation of upstream kinases and transcription factor for CD36 were not affected by CD36 silencing in vivo (Fig. S6). Taken together, our data suggest that prolonged activation of AMPK by berberine in hepatocytes and in mice increased CD36 expression and fatty acid uptake, which were linked to hepatocellular lipid accumulation and fatty liver.

Table 1
Serum biochemical parameters of the mice injected with Ad-shCD36 or control vector followed by intraperitoneal administration of berberine for 7 days.

Characteristics	Ad-shmock BBR		Ad-shCD36 orotic acid	
	Vehicle	BBR	Vehicle	BBR
Body weight (g)	25.2 ± 1.5	22.9 ± 1.6*	25.2 ± 0.7	22.48 ± 1.5*
Liver weight (g)	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Liver/body weight (%)	4.3 ± 0.2	4.8 ± 0.3*	4.8 ± 0.4*	5.3 ± 0.4*
ALT (U/L)	25.9 ± 4.9	26.9 ± 15.9	27.6 ± 7.8	24.0 ± 5.7
AST (U/L)	72.0 ± 16.8	135.1 ± 57.4*	82.1 ± 13.6	143.4 ± 50.8*
GGT (U/L)	1.0 ± 0.1	1.1 ± 0.4	1.0 ± 0.1	1.1 ± 0.4
TG (mg/dL)	78.7 ± 13.3	76.9 ± 13.7	94.9 ± 12.8	94.1 ± 12.9
Total cholesterol (mg/dL)	78.3 ± 8.1	100.3 ± 9.1*	83.4 ± 13.1	85.86 ± 11.2

ALT, alanine transaminase; AST, aspartate transaminase; GGT, γ-glutamyl transpeptidase; TG, triglyceride.
Values are mean ± SD ($n = 7$).
* $p < 0.05$ compared with control group using one-way ANOVA and Tukey's multiple comparisons test.

4. Discussion

Berberine is a well-known AMPK activator and studies describe its anti-diabetic and lipid lowering effects (Kim et al., 2009; Lee et al., 2006). In this study, however, we showed that berberine induces hepatic lipid accumulation due to enhanced expression of CD36 when it is administered to normal mice. Berberine induced AMPK phosphorylation resulting in activation of ERK and C/EBPβ, which led to increased CD36 expression in hepatocytes. In addition, hepatic CD36 expression and lipids were increased in normal diet-fed mice treated with berberine, which was abolished by shRNA-mediated silencing of CD36. The reason for this discrepancy is unclear; however, there is some point of differences. First, all the studies showing pharmacological activity of berberine were conducted in animals on high-fat diet or in those with metabolically challenged conditions such as db/db or ob/ob mice. In these cases, the changes in the fatty acid transport due to the enhanced CD36 expression can be masked by the preexisting conditions and the beneficial effects of AMPK activation. Second, the dose of berberine in our study is relatively higher than that used in other studies, which is

one of the critical points determining berberine toxicity (Kheir et al., 2010). Further studies are needed to clarify the reason for the differences between the beneficial and detrimental effects of berberine.

CD36 plays a key role in developing target organ toxicity in the presence of various environmental chemicals. For example, exposure to dioxin has been linked to increased prevalence of fatty liver in humans. CD36 is a transcriptional target of the aryl hydrocarbon receptor (Lee et al., 2010); therefore, activation of the receptor by dioxin or related chemicals in hepatocytes induces CD36 expression and enhanced free fatty acid uptake leading to steatosis (Angrish et al., 2012). Perfluorooctane sulfonate (PFOS) is an industrial chemical with diverse applications and is thus widely distributed in the environment. PFOS exposure induces the development of hepatotoxicity (Lau et al., 2007). A recent study indicated that PFOS induces hepatic steatosis possibly due to the enhanced expression of CD36 (Wan et al., 2012). Similarly, chronic ethanol consumption increased heart weight and cardiac TG, which is ascribed to increased cardiomyocyte fatty acid uptake by enhanced CD36 expression (Hu et al., 2013). These reports support the idea that expression of fatty acid transporters is closely correlated with the pathogenesis of steatosis and target organ toxicity of chemicals.

During exercise or upon muscle contraction, skeletal muscle increases either the uptake of long-chain fatty acid from the plasma or the lipolysis of intracellular TG for oxidation. One mechanism by which muscle cells upregulate plasma fatty acid uptake is via CD36 trafficking to the sarcolemma. The molecular mechanisms involved in inducing CD36 translocation during exercise and muscle contraction remain unclear. However, the finding that AICAR increased fatty acid uptake in resting-state muscle in wild-type, but not in CD36 knockout mice implies that AMPK induces CD36 translocation (Bonen et al., 2007). However, the role of AMPK in fatty acid uptake is less clear in exercising and contracting muscle (Dzamko et al., 2008). While many studies have investigated the role of AMPK in CD36 translocation during muscle contraction, few studies have examined the effects of AMPK on CD36 expression. Chabowski et al. reported that AMPK activation increased the expression of fatty acid transporters in cardiac myocytes and perfused hearts, but the signaling pathways involved were not elucidated (Chabowski et al., 2006). The regulation of CD36 by AMPK in tissues other than cardiac or skeletal muscle is likewise ambiguous. In macrophages, AICAR did not increase the expression levels of CD36 mRNA and protein (Li et al., 2010).

Several studies examining rodents and humans have revealed a significant link between CD36–fatty acid interactions and the pathogenesis of metabolic syndrome (Griffin et al., 2001; Miyaoka et al., 2001). Gene expression analysis in human liver indicated that the genes involved in fatty acid transport, such as FABP4 and CD36, are upregulated in subjects with high liver fat content (Greco et al., 2008). Therefore, we investigated whether AMPK activation affects CD36 expression and fatty acid uptake in hepatocytes and whether it is linked to the development of fatty liver. Prolonged activation of AMPK by berberine, AICAR or CA-AMPK transduction in hepatocytes and in mice resulted in increased CD36 expression and fatty acid uptake, which were linked to hepatocellular lipid accumulation and fatty liver. Supporting these results, previous studies showed that forced expression of hepatic CD36 was accompanied by a marked rise in hepatic fatty acid uptake, which led to increased hepatic TG storage and secretion (Koonen et al., 2007).

We sought to identify a signaling pathway by which AMPK mediates the transcriptional activation of CD36. AMPK is known to phosphorylate ERK1/2, which is responsible for activating the phosphorylation of C/EBPβ in hepatocytes (Noh et al., 2011). The role of C/EBPβ as a transcription factor is well characterized in adipocyte differentiation. C/EBPβ promotes adipogenesis by inducing the expression of the master adipogenic transcription factor C/EBPα. The C/EBP transcription factor family increases CD36 expression in various cell types with different potency. While C/EBPα increases the expression of CD36 in most cell types, including adipocytes, the transcriptional activation potency of C/EBPβ is dependent on the type of cell (Qiao et al., 2008). Whereas many studies have examined C/EBPβ function on CD36 expression in adipocytes, to our knowledge, no study has reported its role in hepatocytes. We investigated whether AMPK-induced CD36 expression is mediated by this transcription factor. We found that AMPK activation by AICAR, berberine or Ad-CA-AMPK transduction phosphorylates ERK1/2 and subsequently C/EBPβ, which binds to the C/EBP-response element in the CD36 promoter in hepatocytes. In contrast to our results, berberine inhibited the expression of C/EBPα and β, as well as their target gene CD36, in 3T3-L1 adipocytes (Huang et al., 2006). These results suggest the importance of cell type specificity in controlling CD36 expression in response to extracellular stimuli. Ectopic expression of C/EBPβ in NIH 3T3 fibroblasts in the process of differentiation into adipocytes activates the synthesis of PPARγ mRNA, a well-known transcriptional activator of CD36 in various cell types (Feng et al., 2000; Tontonoz et al., 1998; Wu et al., 1996). Recently, it was reported that insulin enhances hepatic expression of CD36 mediated by PPARγ and provokes hepatosteatosis and hepatic insulin resistance (Steneberg et al., 2015). Given the importance of fatty acid transporters in the pathogenesis of fatty liver, further studies are needed to explore the molecular mechanisms regulating CD36 expression in the liver.

Although activation of AMPK has exhibited beneficial effects in several models of metabolic disorders and some types of cancer and thus has been identified as a potential target for drug development, the detrimental effects of AMPK have also been reported in various organs. Transgenic mice overexpressing the constitutively active α1 subunit of AMPK in endothelial cells displayed adverse effects on liver function under obese conditions (Liang et al., 2014). Foretz et al. reported that liver-specific overexpression of constitutively active AMPK α2 led to fatty liver due to the accumulation of lipids released from adipose tissue (Foretz et al., 2005). Therefore, when considering artificial AMPK activation by chemical activators or genetic manipulation for the purpose of reaping beneficial effects, temporal and spatial factors should be considered carefully to prevent adverse effects. To the best of our knowledge, this is the first demonstration that prolonged activation of AMPK in hepatocytes induces transcriptional activation of CD36 and lipid accumulation in hepatocytes. Although many studies have illustrated the role of AMPK in CD36 translocation in contracting muscle, understanding the regulation of CD36 expression and translocation in tissues other than muscle is limited. Further research is needed to elucidate the precise role of AMPK in lipid metabolism in other cell types and tissues.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2016.12.019.

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